AD_____

Award Number: DAMD17-01-1-0355

TITLE: The Role of PRIP in Breast Cancer

PRINCIPAL INVESTIGATOR: Yijun Zhu, M.D.
Janardan K. Reddy, M.D.
Sunil S. Badve, M.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
The Role of PRIP in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0355

6. AUTHOR(S)
Yijun Zhu, M.D.
Janardan K. Reddy, M.D.
Sunil S. Badve, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

E-mail: y-zhu2@northwestern.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Estrogen plays an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER that is a member of nuclear receptor superfamily. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator that is amplified and overexpressed in breast cancer. The proposal was to investigate how the PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. During the second year of the award, we disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We have created heterozygous mice with conditional inactivated PRIP gene which will finally help to reveal the function of this important coactivator in mammary gland.

We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Breast Carcinoma, Estrogen Receptor, Coactivators, Transgenic mouse

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Estrogens play an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER which is a member of nuclear receptor superfamily. Nuclear receptors achieve transcriptional activation through the participation of additional factors known as nuclear receptor coactivators. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator amplified and overexpressed in some breast cancers. The proposal was to investigate how PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. Specifically, transgenic animal models overexpressing PRIP would be developed to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast cancers. PRIP transgenic mouse and PRIP null mutated mouse would be used to examine the role of coactivator PRIP in determining the susceptibility of breast cancer development induced by estrogen and chemical carcinogen. The expression of estrogen responsive genes would be examined to test the role of PRIP as an estrogen receptor coactivator in vivo and identify the estrogen responsive genes that are specifically affected by PRIP overexpression and null mutation. The prevalence of PRIP upregulation and amplification in breast cancers would be examined in breast cancers. The potential polymorphisms in PRIP genes would be identified and the linkage between the identified polymorphisms and breast cancer risk would be elucidated by examining normal female and breast cancer patients. The proposed studies would generate novel information regarding the role of nuclear receptor coactivator in breast cancer development and the importance of the variation of nuclear receptor coactivator in the etiology of breast cancer, and most likely provide new insights regarding prevention and treatment strategies.

BODY

Task 1a. Generation of transgenic animal models overexpressing PRIP to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast tumors.

We made a construct MMTV-PRIP to overexpress PRIP in mammary gland. We then used this construct to develop transgenic mice. Transgenic founders were identified by PCR with primers recognizing the promoter region of the transcription unit. 7 transgenic mouse lineages were identified. The founder mice were bred with C57BL/6 mice to generate F1 progeny. The expression of PRIP transgene in mammary glands from F1 progeny was examined by RNAse protection assay. The expression level of PRIP transgene was about half of that from the endogenous PRIP, which was not high enough for further studies. More transgenic mice will be developed to obtain the mice overexpressing PRIP.

Task 1b. Investigation of the role of coactivator PRIP in the susceptibility for breast cancer development to estrogen and chemical carcinogen using PRIP transgenic mouse and PRIP null mutation mouse.

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum) due in most part to defects in the development of placenta, heart, liver, nervous system and retardation of embryonic growth(1). Transient transfection assays using fibroblasts isolated from $PRIP^{-/-}$ embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of RXRα and to a lesser effect on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

As PRIP null mutation is embryonic lethal, we will mate LoxP-PRIP mouse with MMTV-Cre transgenic mice to obtain mouse with conditional PRIP mutation in mammary gland so that the function of PRIP in mammary gland development and its response to estrogen and carcinogen treatment can be studied.

Task 1c. Examination of the expression of estrogen responsive genes and identification of the estrogen responsive genes that are specifically affected by PRIP overexpresssion and null mutation.

Mammary glands were isolated from *ovariectomized* animals treated with 17β-estradiol or ICI 182,780 for three days. $Poly(A)^+$ RNA was isolated from the mammary glands. cDNA probe was made from poly A^+ RNA for hybridization to Mouse cDNA microarray filters. The filters were then exposed to a phosphor imaging system and analyzed using software to obtain the relative expression level of each gene. We found that estrogen treatment increased the mRNA levels of about fifty genes. GAS6 was picked up for further studies. The induction of GAS6 mRNA expression by estrogen was confirmed by RNAse protection assay. The mammary gland from mice deficient for the GAS6 receptors was examined and found to have increased budding and decreased branching.

Task 2. Determination of the prevalence of PRIP upregulation and amplification in breast cancers.

We have collected about 100 breast cancer specimen from the Department of Pathology, Robert Lurie cancer center. RNAse protection assay revealed that 62 % of the breast cancers have PRIP overexpression. Southern blot studies showed that 10% of the breast cancers have PRIP gene amplification.

Task 3. Investigation of the polymorphism of PRIP gene in Breast cancer.

PCR were performed to cover all exons and 500 bp promoter region of PRIP gene. The PCR products were analyzed by SSCP (single strand conformational polymorphism) and the potential polymorphisms were determined by sequencing. We found polymorphism A to G at nucleotide 289, G to A at nucleotide 2408. We are currently examining these polymorphisms in breast cancer patients and normal females.

KEY RESEARCH ACCOMPLISHMENTS

* PRIP gene was disrupted in mice by homologous recombination. Mice nullizygous for PRIP was found to die between embryonic day 11.5 and 12.5.
* About fifty estrogen-inducible genes including GAS6 were identified. The mammary gland from mice deficient for the GAS6 receptors was found to have increased budding and decreased branching.
* PRIP overexpression and amplification were found in about 60% and 10% of breast tumors, respectively.

REPORTABLE OUTCOMES

(1). **Zhu YJ**, Crawford SE, Stellmach V, Dwivedi RS, Rao MS, Gonzalez FJ, Qi C, and Reddy JK. Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, is a Modulator of Placental, Cardiac, Hepatic and Embryonic development. *J. Biol. Chem.* 278: 1986-1990. 2003

CONCLUSIONS

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We have created heterozygous mice with conditional inactivated PRIP gene which will finally help to reveal the function of this important coactivator in mammary gland. We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.

REFERENCES

(1). **Zhu YJ**, Crawford SE, Stellmach V, Dwivedi RS, Rao MS, Gonzalez FJ, Qi C, and Reddy JK. Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, is a Modulator of Placental, Cardiac, Hepatic and Embryonic development. *J. Biol. Chem.* 278: 1986-1990. 2003

APPENDICES

One article is attached.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 3, Issue of January 17, pp. 1986–1990, 2003
Printed in U.S.A.

Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, Is a Modulator of Placental, Cardiac, Hepatic, and Embryonic Development*

Received for publication, November 12, 2002
Published, JBC Papers in Press, November 22, 2002, DOI 10.1074/jbc.C200634200

Yi-Jun Zhu‡, Susan E. Crawford‡, Veronica Stellmach‡, Rama S. Dwivedi§, M. Sambasiva Rao‡, Frank J. Gonzalez¶, Chao Qi‡, and Janardan K. Reddy‡‖

From the ‡Departments of Pathology and §Pediatrics, Northwestern University, the Feinberg School of Medicine, Chicago, Illinois 60611-3008 and ¶The Laboratory of Metabolism, NCI, National Institutes of Health, Bethesda, Maryland 20892

Nuclear receptor coactivator PRIP (peroxisome proliferator-activated receptor (PPARγ)-interacting protein) and PRIP-interacting protein with methyltransferase activity, designated PIMT, appear to serve as linkers between cAMP response element-binding protein-binding protein (CBP)/p300-anchored and PBP (PPARγ-binding protein)-anchored coactivator complexes involved in the transcriptional activity of nuclear receptors. To assess the biological significance of PRIP, we disrupted the PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (postcoitum) due in most part to defects in the development of placenta, heart, liver, nervous system, and retardation of embryonic growth. Transient transfection assays using fibroblasts isolated from PRIP$^{-/-}$ embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of retinoid X receptor α and to a lesser effect on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

Our understanding of the mechanisms underlying transcriptional activation by nuclear receptors has been advanced by the identification of nuclear receptor coactivators or coregulators that appear to influence embryonic development, cell proliferation, and differentiation (1). These include p160/SRC-1[1] (steroid receptor coactivator-1) family with three members (SRC-1, TIF/GRIP1/SRC-2, and pCIP/AIB1/ACTR/RAC3/TRAM1/SRC3) (2–6), CREB-binding protein (CBP) (7), adenovirus E1A-binding protein p300 (8), peroxisome proliferator-activated receptor-γ (PPARγ)-binding protein (PBP) (9), PPAR-interacting protein (PRIP/ASC-2/RAP250/TRBP/NRC) (10–14) and PPARγ coactivator-1 (PGC-1) (15), among others. Nuclear receptor coactivators contain one or more conserved LXXLL (where L is leucine and *X* any amino acid) signature motif, which has been found to be necessary and sufficient for ligand-dependent interactions with the activation function-2 domain present in the C-terminal hormone-binding region of the nuclear receptors (1, 6). It is generally held that coactivators play a central role in mediating nuclear receptor transcriptional activity by functioning as at least two large multiprotein complexes formed either sequentially or combinatorially (1). The first complex anchored by CBP/p300 and containing p/160 cofactors/SRC-1 cofactors exhibits histone acetyltransferase activity necessary for remodeling chromatin (1, 4, 7, 16), while the second multiprotein complex, variously referred to as TRAP/DRIP/ARC mediator complex, which is anchored by PBP (17–19), facilitates interaction with RNA polymerase II complexes of the basal transcription machinery (1). Deletion of CBP/p300 and PBP genes in the mouse results in embryonic lethality around E11.5 days, indicating that disruption of these pivotal anchoring coactivators affects the integrity of the cofactor complexes, thus altering the function of many nuclear receptors and most likely of other transcription factors (20–24).

Of interest is that the recently identified coactivator designated PRIP/ASC2/RAP250/NRC/TRBP has also been shown to interact with several nuclear receptors and with CBP/p300 and TRAP130 of the TRAP/DRIP/ARC complex (10–14). Thus, PRIP appears to serve as a bridge between the first complex anchored by CBP/p300 and the downstream TRAP/DRIP/ARC mediator complex anchored by PBP. Furthermore, the recently isolated PRIP-interacting protein with RNA methyltransferase activity, designated PIMT (25), forms a complex with CBP, p300, and PBP (26), further attesting to the possibility that two major multiprotein cofactor complexes anchored by CBP/p300 and PBP, respectively, merge into one megacomplex on DNA template (28). Since PRIP and PRIP-binding protein PIMT appear to link the two cofactor complexes under *in vitro* conditions, we have found it necessary to explore the biological function of PRIP by generating mice with PRIP null phenotype. We now demonstrate that PRIP is critical for the embryonic development, since disruption of the PRIP gene in the mouse leads to embryonic lethality around E11.5 to E12.5 days, implying that PRIP (like CBP/p300 and PBP) is also critical for embryonic development and survival.

* This work was supported by National Institutes of Health Grants K08 ES00356 and CA88898 (to Y.-J. Z.), CA64239 (to S. E. C.), CA84472 (to M. S. R.), and GM23750 (to J. K. R.) and by the Joseph L. Mayberry, Sr. Endowment Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Pathology, Northwestern University, the Feinberg School of Medicine, 303 East Chicago Ave., Chicago, IL 60611-3008. Tel.: 312-503-8144; Fax: 312-503-8249; E-mail: jkreddy@northwestern.edu.

[1] The abbreviations used are: SRC-1, steroid receptor coactivator-1; PPAR, peroxisome proliferator-activated receptor; PRIP, PPAR-interacting protein; PBP, PPAR-binding protein; PIMT, PRIP-interacting protein with methyltransferase activity; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element(s); RAR, retinoic acid receptor; RARE, retinoic acid response element; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; TRAP, thyroid hormone receptor-associated protein(s); DRIP, vitamin D_3 receptor-interacting proteins(s); ARC, activator-recruited cofactor; PRIC, PPARα-interacting cofactor complex; ES cells, embryonic stem cells; RT, reverse transcriptase; PCNA, proliferating cell nuclear antigen; MEF, mouse embryonic fibroblast.

MATERIALS AND METHODS

Construction of Targeting Vector—The genomic DNA fragment containing the full-length PRIP gene was isolated from a mouse 129/Sv P1 bacteriophage library (Genome Systems, St. Louis, MO), using polymerase chain reaction with primers 5′-CAATGCAGCCTGTTCCTGT-3′ and 5′-GCTGCTGCATCACCATGAAA-3′ designed from mouse PRIP cDNA sequence (10). A Cre-LoxP system was employed to delete exon 7 from the PRIP gene. For this purpose, we constructed a triple-LoxP PRIP targeting vector to generate a "floxed" mouse PRIP-targeted locus.

Generation of PRIP Null Mice—The targeting vector was linearized and electroporated into HM1 embryonic stem (ES) cells (28). Transfected ES cells were selected in the medium with G418 (200 μg/ml), and the surviving colonies were screened for homologous recombination by PCR with primers P1 5′-CCTACAGCTGCAAGCAAATC-3′ and P2 5′-TATACGAAGTTATGCGGCCG-3′. ES cells with the appropriate PRIP floxed targeted locus were further confirmed by Southern blot analysis (Fig. 1*B*), and the euploid selected ES cells were used for injection into 3.5-day-old blastocysts derived from C57/B6 mouse by the Northwestern University Targeted Mutagenesis Facility to generate chimeric mice. Chimeric male mice were bred with wild type C57/B6 female to produce heterozygous mice, which were then crossed with EIIa-Cre transgenic mice (29) to delete the DNA fragment between LoxP1 and LoxP3. EIIa-cre-mediated recombination occurs early in development (2–8 cells), and mice carrying the allele with deletion were crossed with wild type C57/B6 to achieve germ line transmission. The heterozygous mice with expected deletion were interbred to generate homozygous mutants.

Genotyping of Mice and Embryos—DNA extracted from the tail tips of mice and from the yolk sac of embryos was used for genotyping by PCR. The mice carrying the recombination between LoxP1 and LoxP3 on one allele were identified by PCR with primers P1/P2 and P3 (5′-CGGCCGCATAACTTCGTATA-3′)/P4 (5′-TTCTTCTTCCGAGGCGGTTT-3′). Presence of P1/P2 product, while lacking P3/P4 amplification, indicated the deletion of PRIP gene on one allele. The homozygosity for the deletion was detected by the absence of exon 7 as ascertained by PCR with primers P5 5′-ACGGGCCACCAAATATGATG-3′ and primer P4 (see above) (Fig. 1*C*).

RT-PCR and Western Blots—For RT-PCR, total RNA was extracted from embryos with TRIzol reagent (Invitrogen). Primers 5′-CCTACAGCTGCAAGCAAATC-3′ and 5′-CGAACATGCTGCATGAGCTGA-3′ were used to amplify the region between exon 6 and exon 8 from the PRIP homozygous mutant. To detect PRIP protein in embryos, whole cell lysates were prepared from the embryos by homogenization and probed with anti-mouse PRIP antiserum. The signal was detected by ECL detection system.

Histological Analysis and Immunohistochemistry—Age-matched embryos were fixed in paraformaldehyde or 10% of buffered formalin, embedded in paraffin, serially sectioned at 5-μM thickness in sagittal or transverse planes, and stained with hematoxylin and eosin. Immunohistochemical staining for the localization of proliferating cell nuclear antigen (PCNA) was performed using a standard avidin-biotin-peroxidase complex protocol as described previously (24). Giemsa stain was done using the standard protocol.

Isolation of Fibroblasts from Embryos and Transfection of Primary Fibroblasts—Mouse embryonic fibroblasts (MEF) were isolated from E11.5 embryos and cultured in Dulbecco's modified Eagle's medium with 10% of fetal bovine serum as described (22). For transfection, 2×10^5 of MEFs were plated in six-well plates for overnight culture. The transfections were carried out with LipofectAMINE-2000 Plus reagent according to manufacturer's instruction (Invitrogen). Plasmids pCMV-PPARγ, PPRE-TK-LUC, pCMV-RXR, RXRE-TK-LUC, pCMV-RAR, and RARE-TK-LUC were as described previously (10, 25, 26). β-Galactosidase expression vector pCMVβ was used as a cotransfectant, which served as control for transfection efficiency. Cell extracts were prepared 36 h after transfection and were assayed for luciferase and β-galactosidase activities.

RESULTS

Disruption of PRIP Gene in Mice—We constructed a conditional knock-out allele of PRIP by using the Cre/*loxP* recombination system according to the strategy in Fig. 1*A*. To generate conventional PRIP knock-out mice (in which the gene is permanently inactivated at the germ cell stage), it was necessary to induce recombination between loxP sites. To achieve heterozygosity, PRIP-targeted mice were bred with homozygous Cre transgenic mouse line, EII-cre (29). The EII-cre mice carry

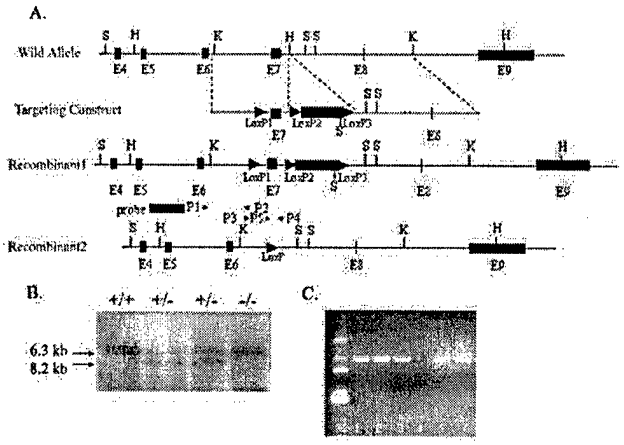

FIG. 1. **Disruption of mouse PRIP gene.** *A*, schematic drawing of the targeting vector and the recombinant alleles. Restriction sites *Spe*I (*S*), *Hin*dIII (*H*), *Kpn*I (*K*), and the location of primers and probes used for ES cell screening and mouse genotyping are indicated. *Dashed lines* show the regions of homologous recombination between the vector and the endogenous gene. *B*, Southern blot analysis of genomic DNAs from ES cells is digested with *Spe*I and hybridized to show PRIP wild type allele (6.3 kb) and recombinant allele (8.2 kb). *C*, DNA from mice derived from heterozygous mating are screened by PCR with primers P4/P5 to detect homozygous deletion of exon 7 (*lane 4*).

the Cre transgene under the control of the adenovirus EIIa promoter and express Cre recombinase only in early mouse embryos (2–8 cell stage), and it induces the recombination between the two loxP sites with the same orientation (29). We detected the expected, all types of recombinants among the offspring (29). The recombination between loxP1 and loxP3 resulted in the deletion of PRIP exon 7 and a reading frameshift to generate a stop codon right after the fusion between exon 6 and exon 8. The chimeras were crossed to wild type mice to produce heterozygous mice carrying one recombinant PRIP allele, and the homozygous mice were obtained from heterozygous mating. By sequencing the RT-PCR products, exon 7 was not found in mRNA transcribed from the recombinant PRIP allele, and the reading frameshift was introduced by the deletion leading to a premature stop codon (data not shown). As the result, only 488 amino acids at the N terminus containing no LXXLL motif can possibly be translated from the mRNA, but no PRIP protein was detected by Western blot analysis (data not shown).

Embryonic Lethality and Growth Retardation of PRIP Null Mice—Among 26 new-born pups, and 54 mice that were 3 weeks old generated from intercrosses between heterozygous PRIP mutant mice, no homozygous mutants were detected. Genotyping the embryos at different stages of gestation showed that no PRIP null embryos survived beyond E13.5 (13.5 days postcoitum). However, heart beating was observed among the majority of viable PRIP null embryos recovered between E11.5 and E12.5 and few were moribund or dead. These observations indicate lethality occurred in a relatively narrow window of time as no viable PRIP null embryos were seen at E13.5. The viable $PRIP^{-/-}$ embryos recovered at E11.5 and E12.5 exhibited clear evidence of growth retardation compared with the wild type and heterozygous littermates. $PRIP^{-/-}$ embryos appeared strikingly different at the gross level at E12.5, they were pale and smaller in size than their $PRIP^{+/+}$ and $PRIP^{+/-}$ littermates (Fig. 2, *A* and *B*). Normally, extraembryonic mesoderm of the yolk sac gives rise to blood and endothelial cells, which form blood islands. The extraembryonic membrane covering PRIP null embryos contained fewer vessels in contrast to wild type yolk sac with its well developed blood vessels (Fig. 2*B*). In addition, superficial vasculature was less obvious in

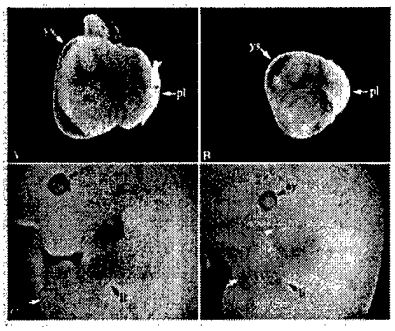

FIG. 2. **PRIP$^{-/-}$ mutants are developmentally retarded.** Wild type (*A*) embryo at E12.5 reveals yolk sac with well developed blood islands, while the PRIP$^{-/-}$ littermate (*B*) is smaller in size with fewer blood islands in its yolk sac (*ys*). *C*, external appearance of eye, blood-enriched liver, and distal part of hind limb with separated fingers (*white arrow*) in wild type embryo. *D*, PRIP$^{-/-}$ embryo shows dilated leaking small vessels (white arrowhead) and few large blood vessels. Pigmented tissue is thinner in the dorsal portion of the eye (*ey*). The liver (*li*) mass is small and pale in the PRIP$^{-/-}$ embryo. Finger separation does not occur in the distal part of hind limb (*white arrow*). Each pair of photographs was taken at the same magnification.

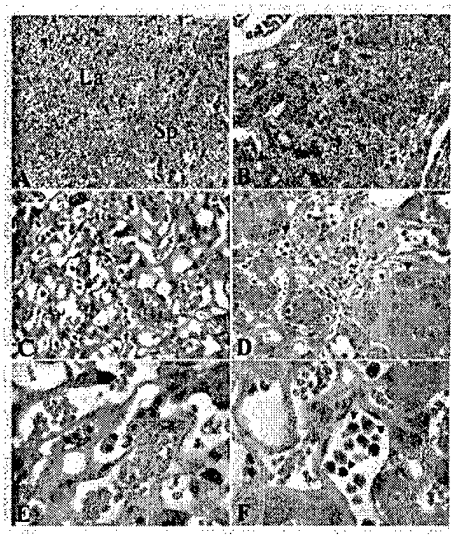

FIG. 3. **Frontal sections of placenta from E12.5 wild type (*A*, *C*, and *E*) and PRIP$^{-/-}$ mutant (*B*, *D*, and *F*) embryos.** *A*, H&E staining of wild type placenta shows labyrinth region (*La*) with extensive well developed fetal vessels surrounded by blood sinuses containing enucleated maternal erythrocytes. The spongiotrophoblast layer (*Sp*) is distinct in wild type placenta. *B*, in PRIP$^{-/-}$ placenta, maternal blood sinuses are generally absent in most areas of labyrinth zone. No organized spongiotrophoblast layer is present. *C* and *D*, close-up view of labyrinth region shows cells with multinucleus clusters in PRIP$^{-/-}$ placenta (*black arrowhead*). Cellularization is less extensive compared with the control. Nucleated fetal erythrocytes in the PRIP$^{-/-}$ placenta (*black arrow*) exhibit irregularly shaped nuclei and have scant cytoplasm compared with control.

PRIP null mutants (Fig. 2*D*). While the liver of wild type embryos was easily visualized by its rich vasculature through the skin, only a pale primitive liver bud was discerned in PRIP$^{-/-}$ embryos (Fig. 2*D*). Failure of palatal shelf to fuse, abnormal finder separations, and developmental abnormalities in brain were detected in PRIP$^{-/-}$ mutants (not illustrated).

Lack of Organized Spongiotrophoblast Layer in PRIP Mutant Placenta—In wild type placenta at E12.5 contains three distinct trophoblast cell structures: the innermost labyrinthine layer, the intermediate spongiotrophoblast layer, and the outermost trophoblast giant cell layer. The labyrinthine zone formed by the fusion of chorion with allantois is composed of extensively branched fetal blood vessels and maternal blood sinuses among strands of diploid trophoblast cells that separate the maternal blood sinuses from fetal blood vessels. In PRIP null placenta, no compact layer of spongiotrophoblast cells was observed between labyrinth zone and trophoblast giant cell layer. Instead, islands of spongiotrophoblast-like cells dissociated from trophoblast giant cell layer and migrating into the labyrinth zone were common occurrence in PRIP$^{-/-}$ placenta (Fig. 3*B*). In wild type placenta blood sinuses are filled with maternal blood cells throughout the labyrinthine layer (Fig. 3, *A*, *C*, and *F*), whereas most of the tortuous vessels in the PRIP$^{-/-}$ placenta were enlarged, ruptured, and generally empty (Fig. 3, *B*, *D*, and *F*). While the chorioallantoic fusion appeared to occur in PRIP mutant placenta, chorionic trophoblast cells clustered in labyrinth had multiple nuclei, and these clusters showed insufficient blood vessel branching. These changes are reminiscent of some of the placental defects observed in PBP null mutants (22, 24). However, changes in PRIP$^{-/-}$ placenta appeared less profound when compared with PBP$^{-/-}$ placenta (24). Nucleated fetal erythroblasts in PRIP$^{-/-}$ placenta had irregular shaped nuclei with very little cytoplasm (Fig. 3*F*). Trophoblast cell proliferation in PRIP null placenta as assessed by PCNA immunostaining was significantly lower than that observed in PRIP$^{+/+}$ placenta (data not shown).

PRIP$^{-/-}$ Embryos Manifest Cardiac Defects—Inefficient pumping by the heart leading to circulation failure is one of the major causes of embryonic lethality during middle gestation. The development of heart requires coordinated differentiation of several embryonic lineages, including the myocytes of myocardium, the endothelial cells of the endocardium, and the cells of the neural crest that form the outflow tract. At E12.5, the heart of PRIP$^{-/-}$ embryos exhibited defects involving all three

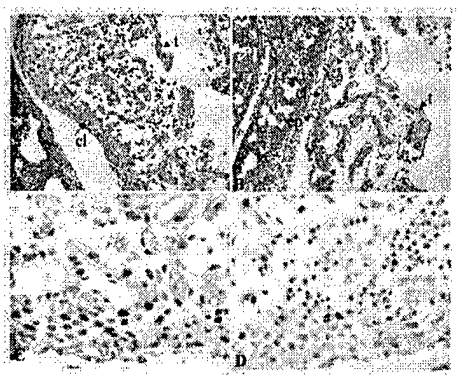

FIG. 4. *A* and *B*, histological analysis of sagittal sections of heart ventricle from E12.5 of wild type (*A*) and PRIP$^{-/-}$ (*B*) embryos. Trabeculation (*t*) is less extensive in PRIP$^{-/-}$ embryo than in the wild type littermate. The compact layer (*cl*) of the mutant is significantly thinner. Epicardium (*ep*) in mutant is separated from underlying myocardium. In some regions of compact layer of PRIP$^{-/-}$, the adhesion of myocytes was disrupted and red blood cells penetrated through myocardium (*arrowheads*). *C* and *D*, PCNA staining for the proliferating cells in ventricles of E12.5 wild type (*C*) and PRIP$^{-/-}$ (*D*) embryos. Fewer *dark brown* PCNA-positive cells are present in the compact layer of PRIP$^{-/-}$ ventricle.

lineages (Fig. 4, *A* and *B*). In PRIP$^{-/-}$ heart, the epicardium, consisting of a single layer of mesothelial cells lining against the compact layer of myocardium, appeared to separate from underlying myocardium and this space is filled with blood cells (Fig. 4*B*). Pericardial space surrounding the heart in mutant was much smaller than their wild type littermate. Unlike the ventricles in wild type heart, which consisted of multicell thick compact layer with well developed trabeculae, the ventricles in PRIP$^{-/-}$ heart only contained one or two cell layers of myocardium (Fig. 4). Cell-cell adhesion among cardiocytes appeared to

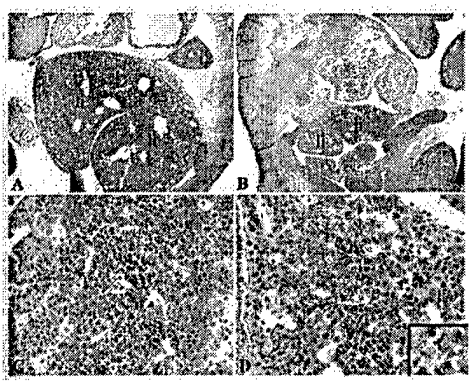

FIG. 5. **H&E staining of liver sections from wild type and PRIP$^{-/-}$ embryos.** At low modification, liver (*li*) in PRIP$^{-/-}$ embryo (*B*) is greatly reduced in size than that of wild type liver (*A*). Liver of mutant contains large numbers of megakaryocytes (*white arrowhead*), fewer of erythrod progenitors (*black arrowhead*), and many cells with apoptotic bodies (*white arrowhead*), in contrast to the normal liver (*C*). The *inset* in *D* shows a closer view of the apoptotic cells in liver PRIP$^{-/-}$ liver.

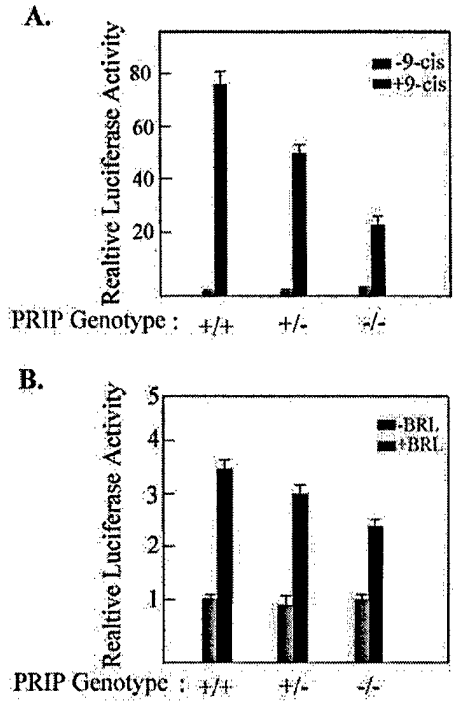

FIG. 6. **Attenuated transcriptional activity of nuclear receptors in PRIP$^{-/-}$ MEFs.** MEFs isolated from E11.5 wild type, heterozygous, and homozygous embryos were cotransfected with 1.5 μg of reporter constructs, 20 ng of vectors expressing nuclear receptors, and 0.1 μg of pCMVβ in the presence or absence of the ligands. The activities of the reporter obtained from transcfection with MEFs from wild type embryos in the absence of ligand was taken as 1. The transfections performed with RXR/RXRE (*A*), PPARγ/PPRE (*B*), and RAR/RARE (not illustrated). The results are the mean ± S.D. of three independent transfections and are normalized to the internal controls of β-galactosidase expression.

be defective, which possibly contributes to leakage of blood cells into epicardial spaces. Cell proliferation in PRIP$^{-/-}$ myocardium was markedly reduced as compared with PRIP$^{+/+}$ myocardium (Fig. 4, *C* and *D*). The failure of ventricular myocardium of PRIP$^{-/-}$ embryos to stratify into the multilayer compact structure required to sustain adequate cardiac function appears similar to that noted in PBP null and PPARγ null mutants (22, 24, 30).

Defective Hepatopoiesis and Hepatic Hematopoiesis in PRIP$^{-/-}$ Mutants—Liver of PRIP null mutants appeared considerably smaller in size when compared with their littermates (Fig. 5, *A* and *B*). The function of the liver at E12.5 days is to become the major site of hematopoiesis so as to gradually replace yolk sac based hematopoiesis. Histological examination of PRIP$^{-/-}$ liver revealed reduction in hepatocyte population and an increase in hepatocyte apoptosis (Fig. 5*D*). A marked decrease in the number of erythroid progenitors was also evident, and these cells had large nuclei with scant minimally hemoglobinized cytoplasm. Liver exhibited large numbers of megakaryocytes.

Differential Reduction of Transactivation by Nuclear Receptors in PRIP$^{-/-}$ Primary Fibroblasts—To assess the impact of loss of PRIP on transcriptional activities of nuclear receptor, we isolated MEFs from PRIP$^{-/-}$ and PRIP$^{+/+}$ embryos. They were used for assaying the transcriptional activities of PPARγ, RXR, and RAR. In wild type MEFs transfected with a RXR expressing vector and RXR-responsive element-linked reporter, the addition of RXR-ligand 9-*cis*-retinoic acid induced marked increase (~76-fold) in the transcription (Fig. 6*A*). In PRIP$^{-/-}$ MEFs, the induction of ligand-mediated RXR transcription was markedly reduced (~3-fold). Transcription assays with PPARγ (Fig. 6*B*) and RARα (data not shown) showed that the influence of PRIP was only modest. These results demonstrated that nuclear receptors require PRIP to achieve their full transcriptional potential, although the contribution of PRIP to the activities of the three nuclear receptors examined here differed somewhat in that PRIP seemed to influence RXR maximally.

DISCUSSION

The nuclear hormone receptors comprise a superfamily of transcription factors that regulates coordinated expression of gene networks involved in developmental, physiological, and metabolic processes (1). Notable among this nuclear receptor superfamily is PPAR subfamily comprising of three isoforms, PPARα, PPARγ, and PPARβ/δ, since these receptors have emerged in recent years as a critical player in regulating energy metabolism. In an effort to understand the factors controlling cell and gene specific transcriptional events initiated by nuclear receptors, the ligand-binding domain of nuclear receptors was used in the yeast two-hybrid screen to identify receptor interacting proteins (2–7, 31). During the past 7 years, more than 25 nuclear receptor coactivators have been cloned raising the issue of redundancy, since these coactivators generally appear promiscuous in their coactivation potential.

To fully appreciate the *in vivo* biological functions of these coactivators, molecular genetic approaches are being increasingly employed. Previous studies have demonstrated that mice lacking SRC-1 or p/CIP/SRC-3 are viable and manifest either partial or full redundancy for certain nuclear receptor actions (32–36). In contrast, deletion of more general coactivators such as CBP/p300 and PBP in mice leads to embryonic lethality implying that these are essential coactivators (20–24). Thus, there appear to be at least two broad classes of coactivators: essential and redundant. Our observations reported here now add coactivator PRIP to the class of essential coactivators because of the embryonic growth retardation, defects in placental, cardiac and hepatic development, and embryonic lethality. Embryonic lethality was noted between E11.5 and E12.5 days with no viable embryos at E13.5. Defects were noted in heart (reduced amount of myocardium and noncompaction), liver (small liver with reduction in hepatocyte population, and hepatocyte apoptosis), and defects in erythropoiesis (reduced hemoglobinization) and placenta (maturation block of trophoblast with vascularization defect). The placental defects, although not as pronounced as those encountered in PBP$^{-/-}$ placenta (22, 24),

nevertheless compromise fetal-maternal exchange of nutrients, growth factors, and oxygen, which may contribute to embryonic growth retardation. The death of embryos at about mid-gestation has been reported in a number of null mutants induced by gene targeting, including PPARγ (30), RXRα (37), PBP (22–24), and N-myc (38).

It should be noted that we isolated both PRIP and PBP using PPARγ as bait in the yeast two-hybrid screen and identified them as nuclear receptor coactivators (9, 10). PBP has since emerged as a central piece in large TRAP-DRIP-ARC-PRIC multiprotein cofactor complex (17–19, 27). Recent studies have established that PBP is indispensable for embryonic development because PBP null mutation leads to embryonic death around E11.5 of mouse development (22–24). PBP null mutation also causes defects in the development of placental vasculature similar to those encountered in PPARγ mutants, supporting the requirement of PBP for PPARγ function *in vivo* (22, 24, 30). PBP null mutants also exhibited cardiac failure because of noncompaction of the ventricular myocardium and resultant ventricular dilatation (22–24). There was also paucity of retinal pigment, excessive systemic angiogenesis, a deficiency in the number of megakaryocytes, and an arrest in erythrocyte differentiation (24). We showed that PBP interacts with GATA family of transcription factors and thus influences the development of vital organ systems (24). Consistent with this view is that the gene encoding PBP is amplified and overexpressed in breast cancer (39). Like PBP, PRIP is also highly amplified/overexpressed in human breast and colon tumors (11), suggesting that both PBP and PRIP by virtue of their coactivating function may augment cell proliferation and neoplastic progression. Finally, the PRIP null MEFs exhibited marked repression of RXR-mediated transcriptional activity as compared with PPAR and RAR. These observations strongly suggest that PRIP has better preference for RXR than other nuclear receptors, and some of the abnormalities noted in PRIP null mutants may be due to inhibition of RXR function. Further studies are needed to examine the role of PRIP in various tissues by generating PRIP conditional null mice.

Addendum—Deletion of the AIB3 (ASC-2) gene has been described recently (40).

REFERENCES

1. McKenna, N. J., and O'Malley, B. W. (2002) *Cell* **108,** 465–474
2. Onate, S. A. Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) *Science* **270,** 1354–1357
3. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J.* **15,** 3667–3675
4. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90,** 569–580
5. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) *Science* **277,** 965–968
6. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387,** 677–684
7. Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855–859
8. Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) *Genes Dev.* **8,** 869–884
9. Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997) *J. Biol. Chem.* **272,** 25500–25506
10. Zhu, Y., Kan, L., Qi, C., Kanwar, Y. S., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (2000) *J. Biol. Chem.* **275,** 13510–13516
11. Lee, S.-K., Anzick, S. L., Choi, J.-E., Bubendorf, L., Guan, X.-Y., Jung, Y.-K., Kallioniemi, O. P., Kononen, J., Trent, J. M., Azorsa, D., Jhun, B.-H., Cheong, J. H., Lee, Y. C., Meltzer, P. S., and Lee, J. W. (1999) *J. Biol. Chem.* **274,** 34283–34293
12. Mahajan, M., and Samuels, H. H. (2000) *Mol. Cell. Biol.* **20,** 5048–5063
13. Caira, F., Antonson, P., Pelto-Huikko, M., Treuter, E., and Gustafsson, J.-A. (2000) *J. Biol. Chem.* **275,** 5308–5317
14. Ko, L., Cardona, G. R., and Chin, W. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6212–6217
15. Lin, J., Puigserver, P., Donovan, J., Tarr, P., and Spiegelman, B. M. (1998) *Cell* **92,** 829–839
16. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
17. Yuan, C. X., Ito, M., Fondell, J. D., Fu, Z. Y., and Roeder, R. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7939–7944
18. Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) *Nature* **398,** 824–828
19. Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) *Nature* **398,** 828–832
20. Yao, T.-P., Oh, S. P., Fuchs, M., Zhou, N.-D., Ch'ng, L.-E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. L., and Eckner, R. (1998) *Cell* **93,** 361–372
21. Kung, A. L., Rebel, V. I., Bronson, R. T., Ch'ng, L.-E., Sieffl, C. A., Livingston, D. M., and Yao, T.-P. (2000) *Genes Dev.* **14,** 272–277
22. Zhu, Y., Qi, C., Jia, Y., Nye, J. S., Rao, M. S., and Reddy, J. K. (2000) *J. Biol. Chem.* **275,** 14779–14782
23. Ito, M., Yuan, C.-X., Okano, H. J., Darnell, R. B., and Roeder, R. G. (2000) *Mol. Cell* **5,** 683–693
24. Crawford, S. E., Qi, C., Misra, P., Stellmach, V., Rao, M. S., Engel, J. D., Zhu, Y., and Reddy, J. K. (2002) *J. Biol. Chem.* **277,** 3585–3592
25. Zhu, Y., Qi, C., Cao, W.-Q., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10380–10385
26. Misra, P., Qi, C., Yu, S., Shah, S. H., Cao, W.-Q., Rao, M. S., Thimmapaya, B., and Reddy, J. K. (2002) *J. Biol. Chem.* **277,** 20011–20019
27. Surapureddi, S., Yu, S., Bu, H., Hashimoto, T., Yeldandi, A. V., Kashireddy, P., Cherkaoui-Malki, M., Qi. C., Zhu, Y.-J., rao, M. S., and Reddy, J. K. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11836–11841
28. Selfridge, J., Pow, A. M., McWhir, J., Maghin, T. M., and Melton, D. W. (1992) *Somatic Cell Mol. Genet.* **18,** 325–336
29. Lee, Y.-H., Sauer, B., Johnson, P. F., and Gonzalez, F. J. (1997) *Mol. Cell. Biol.* **17,** 6014–6022
30. Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A., and Evans, R. M. (1999) *Mol. Cell* **4,** 585–595
31. Lee, J. W., Choi, H. S., Gyuris, J., Brent, R., and Moore, D. D. (1995) *Mol. Endocrinol.* **9,** 243–254
32. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998) *Science* **279,** 1922–1925
33. Qi, C., Zhu, Y., Pan, J., Yeldandi, A. V., Rao, M. S., Maeda, N., Subbarao, V., Pulikuri, S., Hashimoto, T., and Reddy, J. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1585–1590
34. Xu., J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6379–6384
35. Wang, Z., Rose, D. W., Hermanson, O., Liu, F., Herman, T., Wu, W., Szeto, D., Gleiberman, A., Krones, A., Pratt, K., Rosenfled, R., Glass, C. K., and Rosenfeld, M. G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13545–13554
36. Gehin, M., Mark, M., Dennefeld, C., Dierich, A., Gronemeyer, H., and Chambon, P. (2002) *Mol. Cell. Biol.* **22,** 5923–5927
37. Sapin, V., Dolle, P., Hindelang, C., Kastner, P., and Chambon, P. (1997) *Dev. Biol.* **191,** 29–41
38. Charron, J., Malynn, B. A., Fisher, P., Stewart, V., Jeanotte, L., Goff, S. P., Robertson, E. J., and Alt, F. W. (1992) *Genes Dev.* **6,** 2248–2257
39. Zhu, Y., Qi, C., Jain, S., LeBeau, M. M., Espinosa, R., III, Atkins, G. B., Lazar, M. A., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10848–10853
40. Kuang, S.-Q., Liao, L., Zhang, H., Pereira, F. A., Yuan, Y., Demayo. F. J., Ko, L., and Xu, J. (2002) *J. Biol. Chem.* **277,** 45356–45360